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Site-Directed, On-Surface Assembly of DNA Nanostructures

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Abstract: *Two-dimensional DNA lattices have been assembled from DNA double-crossover (DX) motifs on DNA-encoded surfaces in a site-specific manner. The lattices contained two types of single-stranded protruding arms pointing into opposite directions of the plane. One type of these protruding arms served to anchor the DNA lattice on the solid support through specific hybridization with surface-bound, complementary capture oligomers. The other type of arms allowed for further attachment of DNA-tethered probe molecules on the opposite side of the lattices exposed to the solution. Site-specific lattice assembly and attachment of fluorophore-labeled oligonucleotides and DNA–protein conjugates was demonstrated using DNA microarrays on flat, transparent mica substrates. Owing to their programmable orientation and addressability over a broad dynamic range from the nanometer to the millimeter length scale, such supramolecular architecture might be used for presenting biomolecules on surfaces, for instance, in biosensor applications.*

Methods for the site-directed immobilization of biomacromolecules on solid substrates are of paramount importance for fundamental and applied research in biology and medicine. Owing to their high degree of miniaturization and parallelization, microarray techniques are of particular interest for surface-based bioanalytics and biomedical diagnostics. For example, DNA microarrays are nowadays routinely used for genotyping and expression profiling.[1,2] They can also be converted into protein microarrays by the method of DNA-directed immobilization (DDI), wherein surface-bound capture oligonucleotides are used to selectively bind proteins tagged with complementary DNA oligomers.[3] While such micrometer-sized DNA arrays are usually fabricated by so-called "top-down" methods, such as (photo)lithography, ink jet or micro-contact printing, a complementary "bottom-up" approach harnesses the peculiar molecular recognition properties of DNA molecules to self-assemble into nanometer-sized arrays of pre-determined structure and shape.[4] This so-called "structural DNA nanotechnology" has opened the door to a plethora of nanostructures[5] that can also be used as scaffolds for the arrangement of proteins, thus giving access to novel tools for research in materials and life sciences.[6,7]

As a route to bridge lithographically prepared micropatterns with self-assembled DNA nanostructures, mica surface-mediated self-assembly of DNA 3-point-star motifs into 2D nanoarrays[8] and the assembly of DNA double-crossover (DX) tiles into crystal-like arrays by mica-[9,10] or silica-assisted growth[11] have been reported. While these studies provided clear evidence for the feasibility of surface-mediated self-assembly of DNA nanostructures, they lacked control over the spatial orientation of the structures in relation to the substrate. This may indeed be achieved through tailored modification of the assembled constructs with single-stranded tags and their consequent site-directed sorting on patterns encoded with a set of complementary DNA oligonucleotides. We here report, for the first time, on a two-facet method that combines surface-assisted assembly of quasi two-dimensional DNA lattices with site-directed immobilization on DNA microarrays, thus allowing for simultaneous control of nanostructure formation and surface-binding orientation. As shown in Figure 1, DNA tiles were generated bearing complementary cohesive ends to form crystal-like arrays, as well as single-stranded DNA (ssDNA) oligomers to enable hybridization with surface-bound capture oligomers. Programmable orientation of the structures with respect to the substrate allowed for further attachment of DNA-tethered probe molecules on the opposite side of the planar lattices, which are exposed to the solution. This, together with the capability to address the nanostructures with nanometer precision, brings the method a significant step forward towards the goal of merging top-down with bottom-up approaches.

Tile-based systems suitable for assembly of quasi two-dimensional, crystal-like DNA lattices are primarily based on either double-crossover (DX) or so-called 4×4 DNA motifs.[4,12,13] The high structural flexibility of DNA lattices assembled from 4×4 tiles equipped with addressable protruding arms[14] makes these motifs unsuitable for generation of on-surface assembled monolayers. We therefore focused on the known rigid DX motifs, **DX-A** and **DX-B**, that effectively assemble into 2D lattices.[15] To realize the assembly system illustrated in Figure 1, we re-designed these DX tiles to include a single-stranded oligonucleotide protruding perpendicular from the plane of the motif (Supporting Information, Figure S1). The resulting **DX-A1** and **DX-B1** tiles (Figure 1; Supporting Information, Figure S1) hybridize with each other in a way that two consecutive protruding arms (PAs) are separated by 37 base pairs, corresponding to 3.5 helical turns (Supporting Information, Figures S2, S3). Hence, the PAs of neighboring tiles will point in opposite directions on the 2D lattice plane, thus enabling the hybridization-based immobilization through one PA as well as the decoration of a surface-

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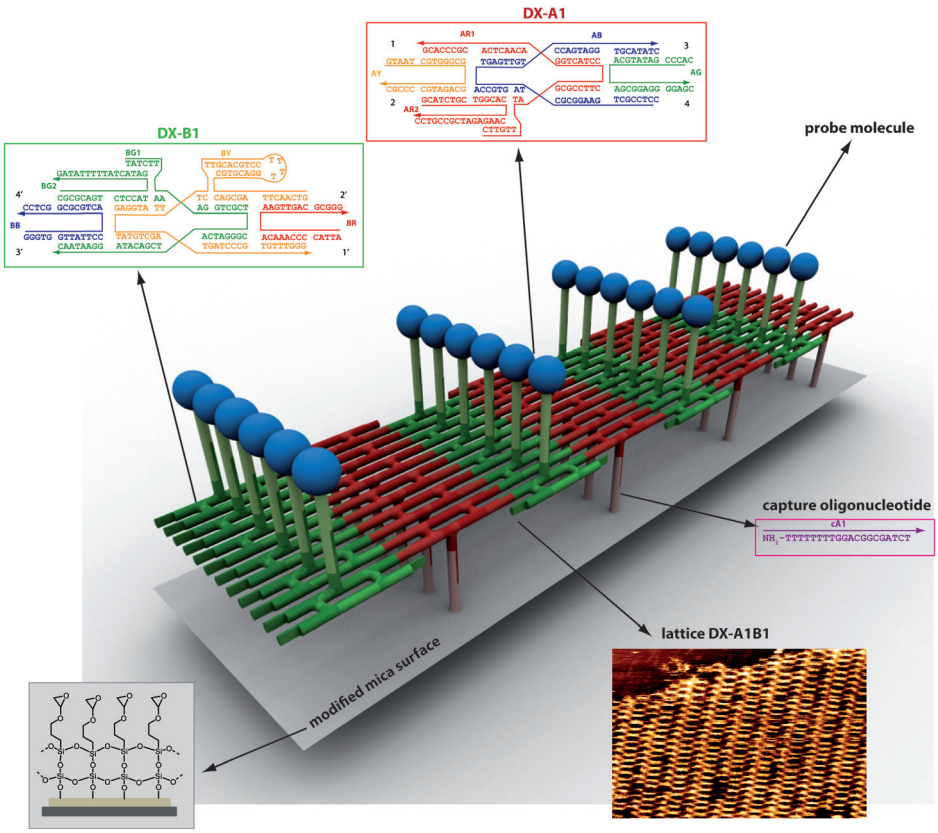

Figure 1. Illustration of surface-bound, quasi two-dimensional DNA lattices (**DX-A1B1**), assembled on capture oligonucleotide-modified mica surfaces. The lattice is formed by periodic assembly of two double-crossover motifs **DX-A1** (red) and **DX-B1** (green). Both DX motifs carry single-stranded oligonucleotides protruding from the lattice plane to facilitate hybridization with surface-bound capture oligomers (via **DX-A1**) or DNA-tethered fluorescent probe molecules (via **DX-B1**). In this study, the site-specific, on-surface assembly was visualized on the nanometer and micrometer length scale by AFM and fluorescence microscopy, respectively.

bound lattice with oligonucleotide-tagged components through hybridization with the other PA. The suitability of these tiles was experimentally confirmed by AFM studies (Figure 2).

In the course of our initial design studies, we also investigated the effect of the ssPA on the stability of individual tiles (Supporting Information, Figure S4) as well as on the assembly of 2D lattices (Supporting Information, Figure S5) using additional constructs, **DX-A2** and **DX-B2** tiles (Supporting Information, Figure S1), which contained unstructured or partially self-complementary PA sequences capable of forming hairpin loops through intramolecular hybridization. These studies indicated that an unstructured 12-mer single-stranded PA slightly destabilized the integrity of the tile while it improved the efficiency of intermolecular hybridization with complementary DNA oligomers, as compared to a hairpin-forming PA. Screening different binary combinations of A and B tiles we observed that the assembly of **DX-A1** and **DX-B1** resulted in large extended 2D lattices (Figure 2A,B, Supporting Information, Figure S5). We also established that both PAs tethered to **DX-A1** and **DX-B1** within the supramolecular lattice of **DX-A1B1** were able to hybridize with complementary DNA probes (Figure 2C; Supporting Information, Figures S6, S7). In brief, the PA sequence of **DX-A1** showed a higher hybridization efficiency for post-assembly binding of oligonucleotides and DNA–protein conjugates than the PA tethered to tile **DX-B1**. As hybridization with surface-bound capture strands was the fundamental prerequisite for successful realization of the on-surface assembly, we chose to employ the better functioning PA of **DX-A1** for this purpose and the weaker binding PA of **DX-B1** for the purpose of post-assembly probe hybridization.

To enable investigation of the on-surface assembly of DX-lattices, solid substrates had to be chosen that met the requirements of both characterization techniques, transparency and low surface roughness to enable fluorescence microscopy and AFM, respectively. To this end, we mounted thin mica sheets on microscopy coverslips and established a procedure for chemical activation of these surfaces by gas phase deposition of 3-glycidoxypropyl-trimethoxysilane (GPTS). The substrates were then functionalized by covalent binding of aminoalkyl-modified ssDNA capture strands (Supporting Information, Figures S8–S11). Importantly, this method enabled the ink-jet spotting of microarrays containing different capture oligonucleotides (spot size ca. 150 μm) which could be readily analyzed by fluorescence microscopy (Supporting Information, Figure S9). Moreover, the surface retained an acceptably low roughness (Supporting Information, Figure S11), thereby enabling AFM visualization of DX lattices assembled on these substrates (Figure 2D; Supporting Information, Figure S12). Indeed, the height of the surface-bound **DX-A1B1** was approximately 2 nm, in good agreement with the expected values for DX lattices.[15] Furthermore, ultrastructural features were clearly distinguishable (that is, the striped patterns of the lattices). AFM analyses of the lattices directly assembled on the modified mica surfaces revealed significantly more, albeit smaller, lattice fragments as compared to lattices assembled in solution. This observation suggests that the capture strands act as nucleation points for the lattice assembly and that hybridization with immobilized capture oligonucleotides competes with the lateral growth of the lattices.

We then tested whether the on-surface assembly of DX lattices can indeed be achieved in a site-directed manner using DNA microarrays (Figure 3). To this end, four different capture oligomers were spotted on the GPTS-modified mica

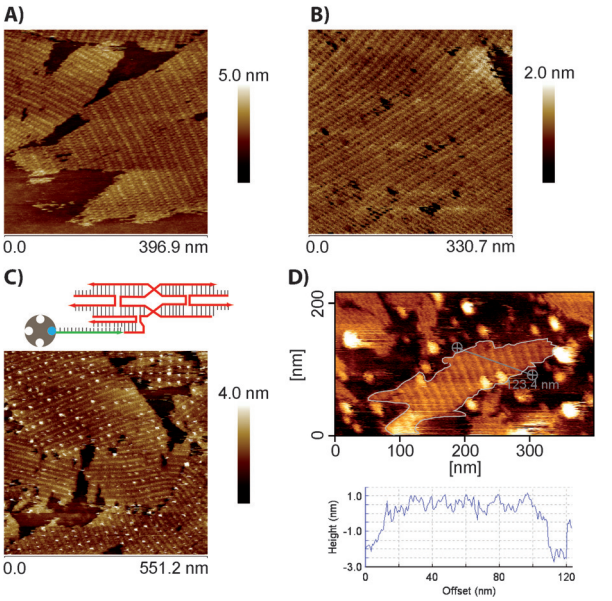

Figure 2. Representative AFM images of lattice **DX-A1B1**. A,B) Lattices assembled in solution using either standard conditions (90 °C→20 °C) or BioCell-compatible conditions (60 °C→20 °C), respectively. Note the typical striped pattern of these lattices originating from the periodicity of the hairpin and/or protruding arms embedded in the lattice (design details in the Supporting Information, Figure S3). C) Post-assembly hybridization with a DNA–protein conjugate complementary to the PA attached to tile **DX-A1** (illustration). Note that the proteins form a regular pattern on the DX-lattice and the protein functionalization of the lattice is almost complete (typically 60–80%; see also the Supporting Information, Figures S6, S7), as indicated by the clearly visible STV stripes alternating with the stripes, originating from the hairpin loops of tiles **DX-B1**. Lower modification yields, visible in small areas at the left side of the image, may stem from insufficient amounts of DNA-protein conjugates. D) On-surface assembled lattice (outlined in gray to increase visibility). Note the typical height of about 2 nm (gray line) and the striped pattern originating from the PAs tethered to tile **DX-B1**, thus indicating successful control of lattice-binding orientation. The irregular artefacts in the surrounding area stem from the chemical modification of the mica surface.

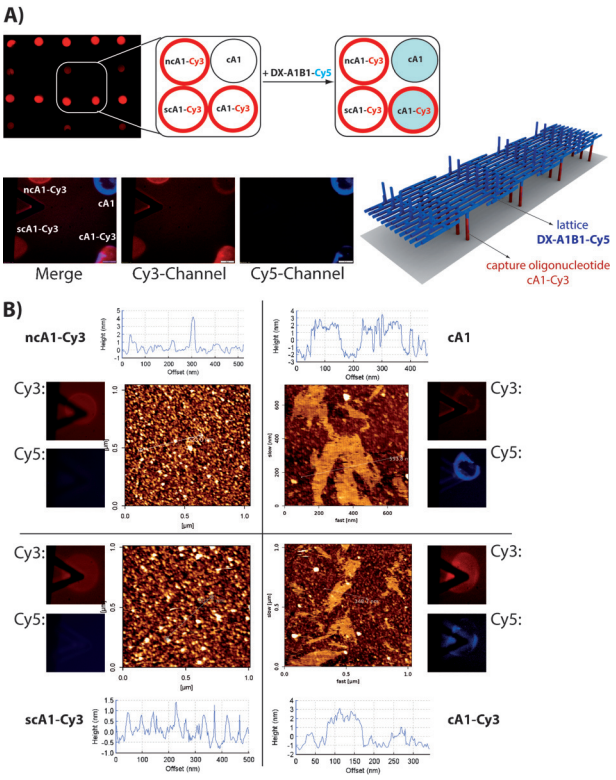

Figure 3. Site-directed, on-surface assembly of **DX-A1B1** lattices using DNA microarrays on GPTS-modified mica substrates. A) Four-feature square block arrangements of the capture oligonucleotides: cA1 and cA1-Cy3 are full-length complementary to the 12-mer ssPA of tile **DX-A1**, scA1-Cy3 contains a 7-mer complementary site, and ncA1-Cy3 is the non-complementary negative control. Capture oligomers are labeled with Cy3 ($\lambda_{Exc}=550$ nm, $\lambda_{Em}=570$ nm) while the DX-lattice contains Cy5-labeled ($\lambda_{Exc}=649$ nm, $\lambda_{Em}=670$ nm) oligomers to enable simultaneous detection by fluorescence microscopy using appropriate filter sets (red and blue channel, respectively). Representative fluorescence micrographs are shown at the bottom. Note that the spots containing unlabeled cA1 show a weak fluorescence in the Cy3 channel due to leakage of the strong Cy5 fluorescence into the Cy3 filter. Spots are about 150 μm in diameter. B) AFM analysis of spots bearing the four different capture oligomers. Each section shows a representative AFM image along with the corresponding height profiles (blue curves) and fluorescence micrographs (note the triangular shaped AFM cantilever visible at the left side of the spots).

substrate in four-feature square block arrangements in a way that the entire 1×1 cm^2 piece of mica was covered with 30×30 spots, each with a diameter of about 150 μm (Figure 3, Supporting Information, Figure S9). Specifically, the four square blocks contained capture oligomers cA1 and cA1-Cy3, both of which are fully complementary to the 12-mer ssPA of tile **DX-A1**. Moreover, capture oligomer scA1-Cy3, which contains a shortened 7-mer complementary recognition site, was used to estimate the influence of sequence length, and fully non-complementary capture oligomer ncA1-Cy3 was used as a negative control. To minimize steric hindrance on the surface, all capture oligomers contained a dT_8 spacer sequence in between the recognition site and the 5′-alkylamino group bound to the substrate (Table S1).

On-surface assembly was carried out in a temperature-controlled fluid cell of an AFM instrument (BioCell of the Nanowizard 3 AFM, JPK) equipped with an inverse fluorescence microscope. The latter was used for the detection of immobilized capture probes in the Cy3 channel (red spots in Figure 3) and DX lattices, which were labeled with Cy5 (blue spots) at the single-stranded PA of tile **DX-B1**. Owing to instrumental limitations of the BioCell, assembly of **DX-A1B1** lattices on the surface of DNA-patterned mica substrates was carried out by cooling the mixture of the nine DX-forming oligonucleotides from 60 °C→20 °C with a rate of −0.1 °C min^{-1}. Because this protocol varies from the standard procedure (90 °C→20 °C, −0.1 °C min^{-1}),[15] we confirmed its suitability for the assembly of **DX-A1B1** lattices in homogeneous solution (Figure S5).

It is clearly evident from Figure 3 that on-surface assembly of the DX lattices occurred in a highly site-specific fashion: strong Cy5 signals were detectable on the spots bearing the complementary capture oligomer (cA1) whereas no Cy5 fluorescence could be detected either on the negative control (ncA1), the short 7-mer capture (scA1), or on the

surrounding unfunctionalized mica surface. Owing to the instrumental coupling of fluorescence microscopy and AFM, we were able to precisely analyze the surface of specific spots by AFM imaging (Figure 3B). These measurements proved the formation of nanostructured DNA lattices on surfaces containing complementary capture oligomers cA1 while no such structures were present in spots containing the negative control or the 7-mer capture (ncA1 or scA1, respectively).

As described above, the on-surface assembled DX-lattices were anchored to the mica through the PA of tile **DX-A1**. Therefore the PA tethered to tile **DX-B1** should still be available for hybridization with complementary oligonucleotide-tagged probe molecules. To experimentally verify this concept, we tested the post-assembly modification of surface-immobilized DX-lattices with either a fluorescein (Fsc)-labelled oligonucleotide (Supporting Information, Figures S13, S14) or a complementary DNA-protein conjugate (Figure 4; Supporting Information, Figure S15). The DNA–protein conjugate was made from STV and contained one oligonucleotide and the fluorescent dye Atto488, which enables fluorescence microscopy detection orthogonal to the Cy3 and Cy5 labels of the capture strands and DX lattice, respectively. Again, four-feature square block arrangements of capture oligonucleotides were used for these experiments, except that the short 7-mer capture (scA1) was omitted and, instead, positive control oligomer cA1 was spotted in duplicate (Figure 4). Following on-surface assembly of lattice **DX-A1B1**, the complementary DNA-STV probe was allowed to hybridize with the surface-bound DX-lattice and the resulting assemblies were analyzed by fluorescence microscopy. It is clearly evident from Figure 4D that Atto488 signals were only present in spots which contained the cA1 capture, complementary to the **DX-A1B1** lattice. While these fluorescence measurements clearly indicated that probe immobilization occurred exclusively through on-surface assembled DNA nanostructures, exact topographic characterization by AFM could not be achieved (Supporting Information, Figure S16). Similar results were obtained with on-surface assembled **DX-A1B2** lattices (Supporting Information, Figures S14, S15), thereby indicating that the system

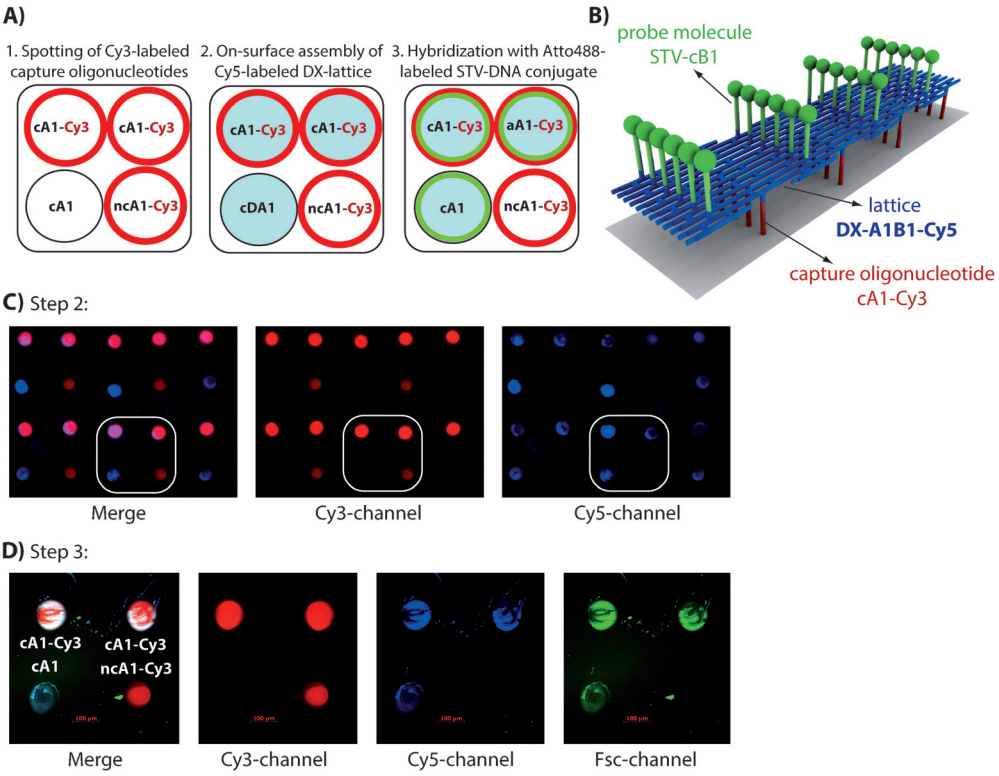

Figure 4. Post-assembly modification of on-surface assembled lattice **DX-A1B1-Cy5**. A) Flow chart of the experiment, and (B) schematic illustration of the system. The spotted four-feature square block arrangements of the capture oligonucleotides (step 1) are similar as described for Figure 2, except that cA1 was spotted in duplicate. The DX-lattice was anchored to the mica surface via the PA tethered to tile **DX-A1** (step 2, C), thus leaving free the PA tethered to tile **DX-B1** for hybridization with a complementary DNA-STV conjugate (step 3, D) fluorescently labeled with Atto488 ($\lambda_{Exc} = 501$ nm, $\lambda_{Em} = 528$ nm). Note that binding of the latter is detectable in the Fsc channel and occurred only in spots which contain the cA1 capture oligomer, thus being indicative for the site-specifically on-surface-assembled DX lattice.

described here can be tuned by design of tailored DNA nanostructures.

In conclusion, we demonstrated that DNA nanostructures can be site-specifically assembled on DNA-encoded surfaces. While the on-surface assembly of DNA oligomers has previously been suggested as a route to bridge top-down micropatterning of solid supports with bottom-up self-assembly of nanostructures,[8,11] the use of DNA-modified surfaces described herein adds control over the directionality of binding to the substrate. Specifically, the top-down patterning of solid supports with DNA capture oligonucleotides can be combined with bottom-up self-assembly of DNA nanostructures to open the door to fabrication of tailored surfaces bearing sophisticated patterns of biomacromolecules over a broad dynamic range from the nanometer to the micro- and millimeter length scale.

The work described here developed a flat and transparent mica-on-glass substrate that enables the detailed analysis of the on-surface assembly processes through atomic force microscopy and fluorescent imaging. With this advance, we believe that potential applications of surface-assembled nanostructures, for instance as tools in life sciences, come into realistic perspective because DNA-encoded microstructures have proven their utility as devices for cell biology and biosensing.[3]

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[1] M. C. Pirrung, *Angew. Chem. Int. Ed.* **2002**, *41*, 1276; *Angew. Chem.* **2002**, *114*, 1326.
[2] A. Sassolas, B. D. Leca-Bouvier, L. J. Blum, *Chem. Rev.* **2008**, *108*, 109.
[3] R. Meyer, S. Giselbrecht, B. E. Rapp, M. Hirtz, C. M. Niemeyer, *Curr. Opin. Chem. Biol.* **2014**, *18C*, 8.
[4] N. C. Seeman, *Nature* **2003**, *421*, 427.
[5] N. C. Seeman, *Annu. Rev. Biochem.* **2010**, *79*, 65.
[6] C. M. Niemeyer, *Angew. Chem. Int. Ed.* **2010**, *49*, 1200; *Angew. Chem.* **2010**, *122*, 1220.
[7] J. Fu, M. Liu, Y. Liu, H. Yan, *Acc. Chem. Res.* **2012**, *45*, 1215.
[8] X. Sun, S. Hyeon Ko, C. Zhang, A. E. Ribbe, C. Mao, *J. Am. Chem. Soc.* **2009**, *131*, 13248.
[9] S. Hamada, S. Murata, *Angew. Chem. Int. Ed.* **2009**, *48*, 6820–6823; *Angew. Chem.* **2009**, *121*, 6952.
[10] J. Lee, S. Hamada, S. U. Hwang, R. Amin, J. Son, S. R. Dugasani, S. Murata, S. H. Park, *Sci. Rep.* **2013**, *3*, 2115.
[11] J. Lee, S. Kim, J. Kim, C. W. Lee, Y. Roh, S. H. Park, *Angew. Chem. Int. Ed.* **2011**, *50*, 9145–9149; *Angew. Chem.* **2011**, *123*, 9311.
[12] U. Feldkamp, C. M. Niemeyer, *Angew. Chem. Int. Ed.* **2006**, *45*, 1856–1876; *Angew. Chem.* **2006**, *118*, 1888.
[13] N. C. Seeman, *Mol. Biotechnol.* **2007**, *37*, 246.
[14] B. Saccà, C. M. Niemeyer, *Small* **2011**, *7*, 2887.
[15] E. Winfree, F. Liu, L. A. Wenzler, N. C. Seeman, *Nature* **1998**, *394*, 539.

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